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Magnetic bead-based salivary peptidome profiling analysis during orthodontic treatment durations

Jieni Zhang [a], Shaonan Zhou [a], Hui Zheng [a], Yanheng Zhou [a], Feng Chen [b,*], Jiuxiang Lin [a,*]

[a] Department of Orthodontics, School of Stomatology, Peking University, Beijing 100081, PR China
[b] Central Laboratory, School of Stomatology, Peking University, Beijing 100081, PR China

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ABSTRACT

Orthodontic treatment induces various biological responses, including tooth movement and remodeling of alveolar bone. Although some studies have investigated the contribution of orthodontic procedures to changes in saliva conditions, little is known about the effects of different treatment durations on the saliva proteome. To identify the discriminating protein profiles in unstimulated whole saliva of orthodontic patients with different treatment durations, we used matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) combined with magnetic bead, and peptide mass fingerprints were created by scanning MS signals. Saliva samples from 40 patients (10 in each of four groups: the group without an appliance and groups under treatment for 2, 7, and 12 months) were analyzed. The results showed eight mass peaks with significant differences. Furthermore, mass peak intensities at proteins 1817.7, 2010.7, 2744 and 2710.2 Da represented a steady time-dependent increasing trend, whereas protein 4134 Da exhibited a decreasing tendency. Differential expression of the peptidome profile also occurred in the multiple comparisons, and we established a fitting model. Thus, the potential discriminating biomarkers investigated in this study reflected the complicated changes in periodontal tissues during orthodontic treatment and indicated dynamic interactions between orthodontic treatment and the saliva proteome. The results provide novel insights into alterations in salivary proteins due to different orthodontic treatment durations and may lead to the development of a therapeutic monitoring strategy for orthodontics.

1. Introduction

Orthodontic treatment employs the principle that orthodontic forces activate the cells and extracellular matrix of periodontal tissues to facilitate tooth movement [1]. Thus, the tooth position changes due to the response of various mineralized and non-mineralized tissues, such as periodontal ligaments, gingiva, and alveolar bone, to orthodontic treatment. Various biological responses occur during prolonged orthodontic treatment [2]. With the application of orthodontic force, the associated blood vessels, neural elements, and cells continue to perform sequential activities, indicating that periodontal circumstances differ as the orthodontic procedure proceeds. These changes are accompanied by relevant factors of bone metabolism, root resorption, and inflammation.

The application of orthodontic force creates regions with different load–strain relationships. Bone resorption is dominant in the region where pressure is applied in the direction of tooth movement, and bone formation is dominant in the reverse tensile region [3]. Thus, following tooth movement, the cellular and molecular events linking the osteoclastic or osteoblastic activities may fluctuate [4]. Bone deposition-related factors, such as tumor growth factor-β, bone morphogenetic proteins, growth factor, alkaline phosphatase, bone resorption factor receptor activator of nuclear factor-κB, receptor activator of nuclear factor-κB ligand, osteoprotegerin, and tartrate-resistance acid phosphatase, may change under certain conditions [5]. Other molecules that have been associated with orthodontic-induced inflammatory root resorption may differ, such as interleukin(IL)-1β, IL-6, and tumor necrosis factor-α [6]. Furthermore, these factors are not stably expressed throughout the entire orthodontic procedure. Studies have shown that the extent of root resorption differs by treatment duration [2]. The features of alveolar bone remodeling also change as tooth movement occurs.

A biomarker is an informative signal and can be a hormone, cytokine, growth factor, inflammatory factor, or any other factor related to a specific condition. The specificity and sensitivity of a

* Corresponding authors. Address: Central Laboratory, School of Stomatology, Peking University, #22 Zhongguancun South Road, Haidian District, Beijing 100081, PR China. (F. Chen), Department of Orthodontics, School of Stomatology, Peking University, #22 Zhongguancun South Road, Haidian District, Beijing 100081, PR China. Fax: +86 010 82195536 (J. Lin).

E-mail addresses: sd88383511@126.com (J. Zhang), shaonanzhou@gmail.com (S. Zhou), zhenghui_cn@163.com (H. Zheng), yanhengzhou@gmail.com (Y. Zhou), moleculecf@gmail.com (F. Chen), jxlin@pku.edu.cn (J. Lin).

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biomarker describe its usefulness in diagnosing a specific condition or predicting its progress [7]. An effective biomarker should be measurable in accessible body fluid, such as serum, urine, or saliva [8]. Saliva, an attractive human biological fluid, contains a complex balance of secretions from the major and minor salivary glands as well as constituents from gingival crevicular fluid, oral microflora, food debris, and desquamated epithelial cells [9]. Therefore, saliva contains abundant proteins, peptides, small molecules, and other compounds. Saliva-based proteomics/peptidomics research has become a recent focus due to its non-invasiveness, convenience, and low cost. Biomarkers in saliva have been identified by recent improvements in mass spectrometry (MS)-based methods, including surface-enhanced laser desorption/ionization time-of-flight MS and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS [7]. MALDI-TOF MS has become the most suitable strategy due to its relative convenience, high sensitivity, and throughput potential. Furthermore, the weak cation exchange (WCX) approach used prior to MALDI-TOF MS analysis is effective for separating low-molecular-mass range peptides (1–10 kDa) from complex body fluids [10].

In this study, we compared differences in protein mass peaks from orthodontic patients with different treatment durations by MALDI-TOF MS using a magnetic bead-based peptidome analysis of saliva samples. We aimed to identify a panel of specific biomarkers for differential expression.

2. Materials and methods

2.1. Ethics statement

This study was approved by the Peking University Biomedical Ethics Committee. Adult subjects and parents of pediatric subjects signed an informed consent form before the start of research.

2.2. Subjects

Patients seeking treatment at the orthodontic department at the Stomatology School of Peking University were recruited in November 2011. All 40 study subjects were systemically healthy, and those who presented with caries, gingivitis, or periodontitis; diseases of the oral mucosa; or oral cancers were excluded.

In this study, the orthodontic treatment plan for all subjects which showed similar malocclusion was to extract four premolars using the same fixed appliance, and the whole procedure of orthodontic treatment for the extraction cases that were selected consisted primarily of aligning, leveling, and space closing. Then, 0, 2, 7, and 12 months were selected as corresponding to before-treatment, aligning, leveling, and space closing, respectively. The four groups were matched in terms of gender ($P = 1$) and ethnicity ($P = 1$). The mean age and gender distribution in each group are shown in Table 1.

All patients were asked to maintain good oral hygiene during treatment and were counseled regarding oral hygiene and tooth brushing before treatment and at each visit.

2.3. Saliva collection and processing

All individuals were asked to rest for 15 min before saliva collection at 8:30 am, and not to eat or drink after dinner the previous evening or to brush their teeth on the collection day morning. The subjects sat in an upright position in a quiet room, and were required to put the tip of their tongue against sublingual caruncle without straining. Thus, the saliva, which was received into a paper cup for the first 5 min, could run from the mouth, and we collected the spontaneous saliva flow using a 50-mL centrifuge tube until 6 ml was collected. During the collection procedure, they were asked not to speak. Immediately after collection, the 6 ml unstimulated whole saliva samples were kept on ice and then centrifuged at 9000*g* for 7 min at 4 °C to remove insoluble materials, cells, and debris. The supernatant of each sample was obtained, 1 mM ethylene diamine tetraacetic acid (Sigma, St. Louis, MO, USA) and 1 mM phenylmethyl sulfonylfluoride (Sigma) was added to inhibit protease activity. Protein concentration was measured using the Lowry assay method and the ELx808 Protein Assay (BioTek, Hercules, CA, USA). Supernatants were kept at −80 °C for further analysis.

2.4. Reagents and instruments

The WCX magnetic bead kit (SPE-C; Bioyong Tech, Beijing, China), alpha-cyano-4-hydroxycinnamic acid (HCCA), MALDI-TOF MS (Bruker Bio-sciences, Bremen, Germany), 100% ethanol (chromatographic grade), and 100% acetone (chromatographic grade) were freshly prepared.

2.5. WCX fractionation and MALDI-TOF MS analysis

The suspension in the WCX magnetic bead kit was mixed by shaking. After eluting and beating, the magnetic beads were separated from the protein, and the eluted peptide samples were transferred to a 0.5-mL clean sample tube for further MS analysis.

Five microliters of HCCA substrate solution (0.4 g/L, dissolved in acetone and ethanol) and 0.8–1.2 μl of elution were mixed. Then, 0.8–1.2 μl of this mixture was applied to a metal target plate and dried at room temperature. Finally, the prepared sample was analyzed by MALDI-TOF MS. A range of 1000–10,000 Da peptide molecular weight was collected, and 400 shots of laser energy were used. Peptide mass fingerprints were obtained by accumulating 50 single MS signal scans.

2.6. Statistical analysis

An analysis of variance was used to identify differences in protein levels among saliva samples from the four groups. The *t*-test and *w*-test were used for multiple comparisons between groups. Data were analyzed using the BioExplorer statistical package (BioyongTech). A p-value < 0.05 was considered significant.

Table 1
Demographic information for subjects in different treatment groups.

Treatment duration (months)	Sample size	Age		Sex (male:female)
		Mean	SD	
0	10	23.2[a]	1.3165	5:5
2	10	13.8	2.2509	5:5
7	10	13.2	1.7512	5:5
12	10	13.6	1.5776	5:5

[a] The average age in the no-appliance group differed from that in the other three groups because of sample collection difficulties.

3. Results

The entire mass spectra of the extracted peptide samples from 40 subjects in the four groups were obtained by MALDI-TOF MS (Fig. 1). The peaks in saliva peptidome fingerprints were characterized in each patient by showing the maximum intensity within a certain *m/z* range. The majority of the peptide molecular weight was 1000–7000 Da. Then, the peaks among the mass spectra were quantified and compared.

An average of 144 protein mass peaks were detected when the four groups were compared. Peak intensities differed significantly for eight peptides (2010.7, 2744.8, 2481.4, 1817.7, 4134, 1425.4, 2710.2, and 1306.1 Da; Table 2). The mass peaks of proteins 1817.7, 2010.7, 2710.2, and 2744.8 Da represented a steady time-dependent increasing trend among the four groups. Patients in the group without appliances showed the lowest mass peaks for these four peptides, whereas patients who had been in treatment for 2 months showed higher peaks, which was similar to the next two groups. This trend was reversed among the four groups at protein 4134 Da and showed a steady time-dependent decrease (Figs. 2 and 3).

The multiple comparison test found the most significant difference between the group without appliances and the group under orthodontic treatment for 12 months (Table 3). Meanwhile, the two peptides (2744.8 and 2010.7 Da) exhibited the most significant difference ($p < 0.01$) when the four groups were compared, and the fitted results of the other combinations were not so good as it. Thus, we chose these two peptides to establish a fitted curve (Fig. 4). The shape showed the well-separated locations of the samples from the two groups, indicating that the fitting results were satisfactory.

Table 2
Significant ($p < 0.05$) *m/z* values discriminating samples from the four groups.

Mean *m/z* value	*P*-value	Tendency[a]
–	7.97E-04	↑
2744.8	0.007	↑
4811.9	0.017	
1817.7	0.020	↑
4134	0.028	↓
2326.3	0.038	
2710.2	0.042	↑
1425.4	0.045	

[a] Tendency refers to the trend in the intensity of *m/z* values among the four groups: ↑, steady time-dependent increasing trend among the four groups; ↓, decreasing trend among the four groups.

Moreover, the successfully identified peptides at 2010, 2744.8, 1817.7, and 4134 Da were predicted to be Apolipoprotein E (apoE) precursor, clusterin (CLU) precursor, type I cytoskeletal 13, and SERPINA1 (PRO2275), respectively.

4. Discussion

In the present study, we used MALDI TOF MS-based proteomic methods and WCX magnetic beads to examine 40 saliva samples. We found significant differences in mass spectra peak intensities among the four different groups according to orthodontic treatment duration, indicating that the current method could be used to

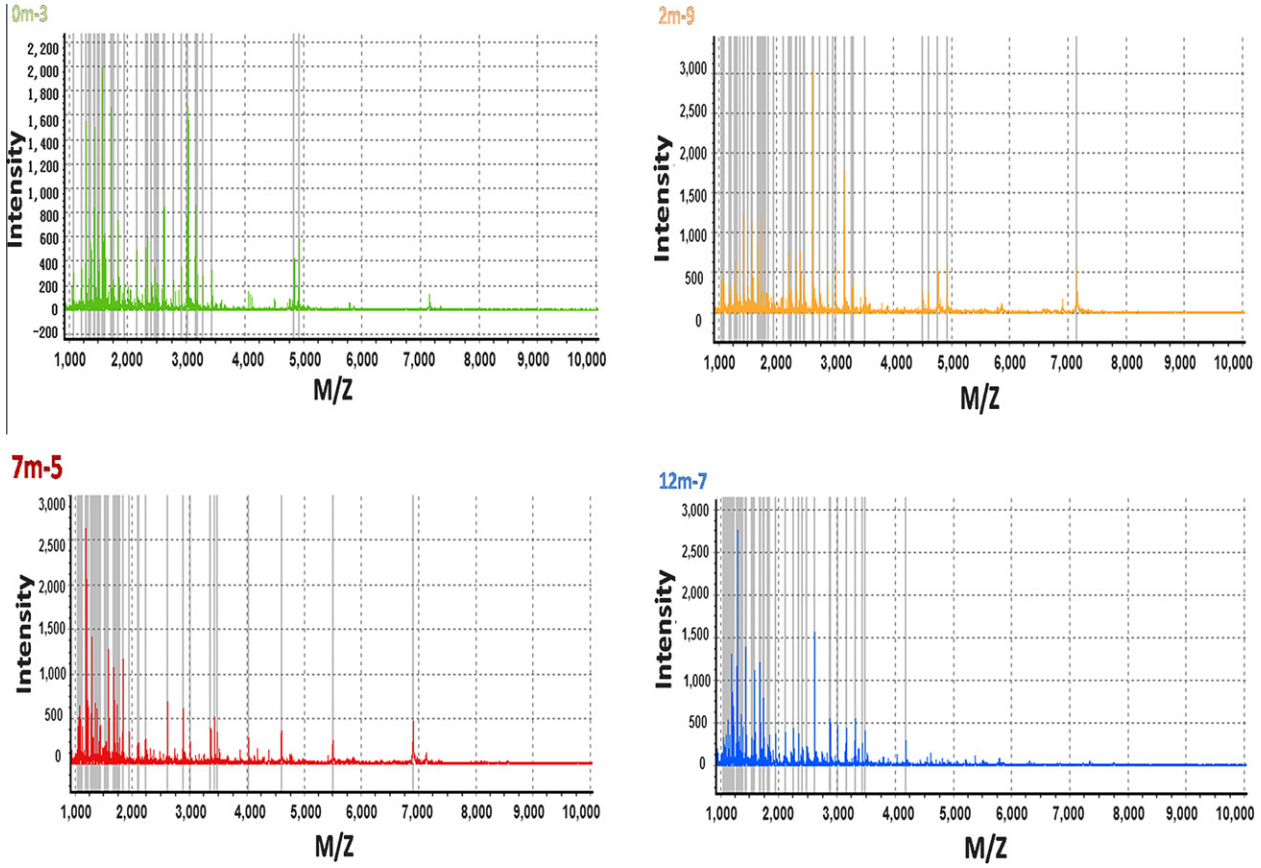

Fig. 1. Complete mass spectra in the range of 1000–9000 Da, showing the peptide fingerprints of a saliva sample from a single patient in each group: no fixed appliance (green curve); 2-month group (orange curve); 7-month group (red curve); 12-month group (blue curve). m/z, mass-to-charge ratio. (For interpretation of the references to colours in this figure legend, the reader is referred to the web version of this article.)

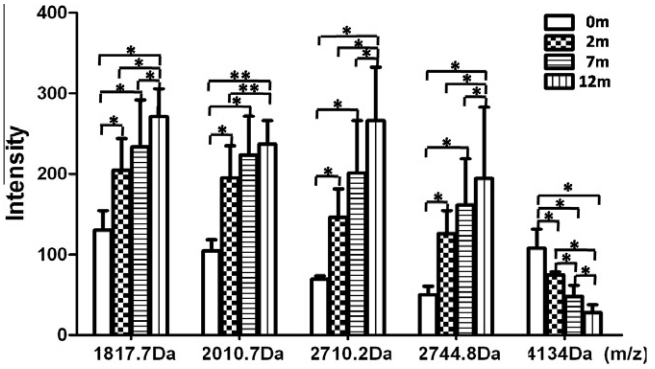

Fig. 2. Column view of the mass spectra from the four different groups, showing an increasing trend in peak intensity at 1817.7, 2010.7, 2710.2, and 2744.8 Da, and a decreasing trend at peak 4134 Da. ($^{*}p < 0.05$; $^{**}p < 0.01$).

analyze peptide profile features during orthodontic treatment. Orthodontic treatment is based on the principle that force from an appliance causes the remodeling of periodontal tissue and tooth movement. During this complex process, alveolar bone metabolism, root resorption, and tissue inflammation also occur. These physiological or pathological conditions are usually accompanied by changes in various elements or biomarkers reflecting the inner mechanism [11]. Thus, this method provides a new tool to analyze the mechanism of orthodontic tooth movement.

Saliva is a vital body fluid that contains more than 2000 kinds of proteins, which maintain many biological functions [12]. More than 2000 low-molecular-weight peptides comprise the salivary peptidome. In addition to the main origin of proteolysis, the peptidome accounts for 40–50% of secreted proteins [13]. As saliva has a large array of informative components and collection is non-invasive, simple, and low-cost, interest in the use of saliva as a detection strategy for oral and systemic conditions has increased [14]. Proteomics/peptidomics and its related techniques have advanced significantly over the last two decades [7], and the saliva-based proteomic approach enables the exploration of diagnostic biomarkers. Protein profiling methods, such as two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) for separation and MS for identification, have been used to explore various conditions and certain disorders, such as breast cancer [15], Sjögren's syndrome [16], rheumatoid arthritis [17], and oral pathologies such as oral cancer [18,19], dental caries [20], cleft palate [21], and periodontitis [22]. These studies have suggested some specific markers, such as inflammatory mediators, which play an important role in disease.

Moreover, advanced MS-based proteomic techniques are required for proteomic analysis. The MALDI-TOF MS technique used in this study is sensitive for recognizing a large mass range, and the

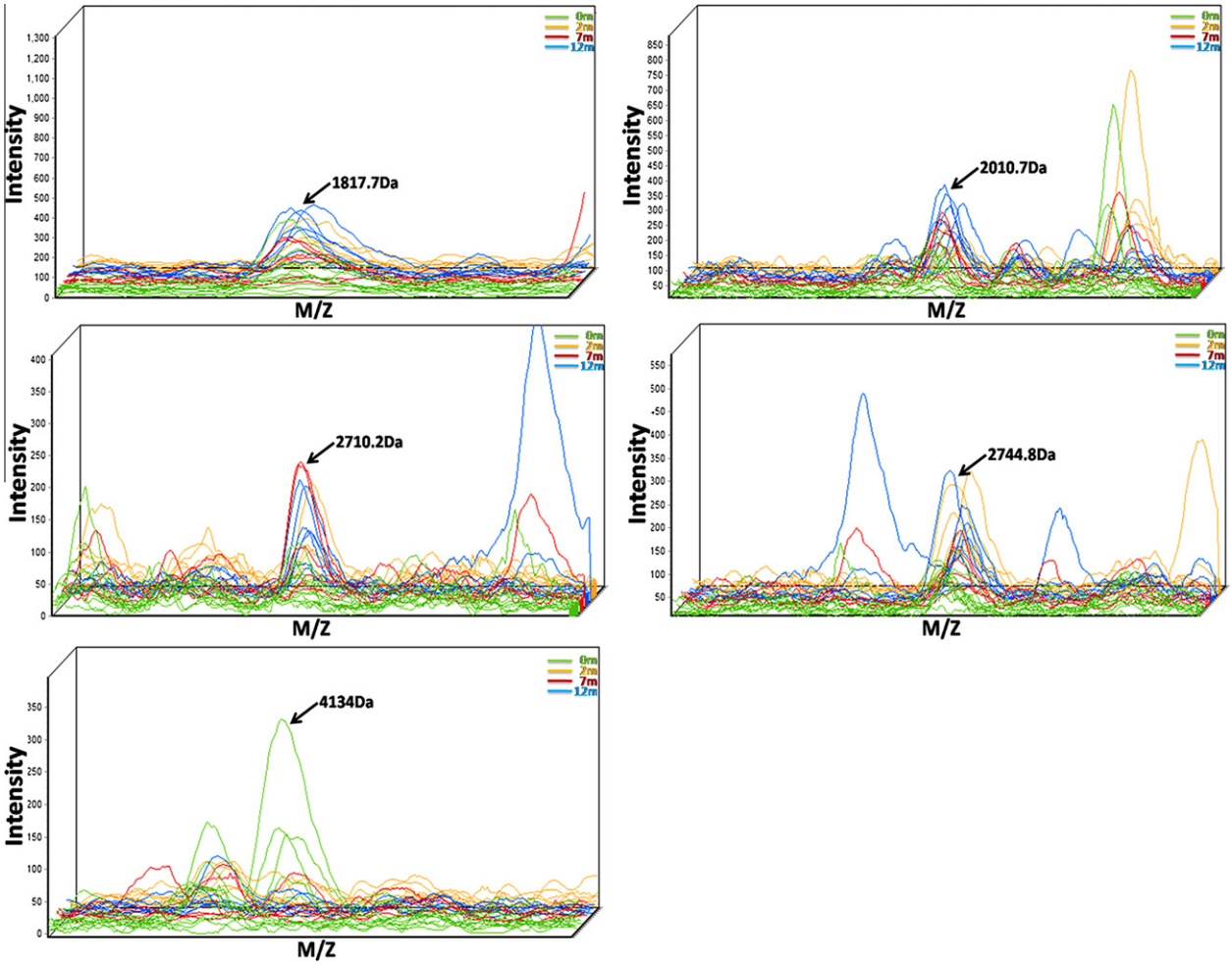

Fig. 3. Three-dimensional *m/z* ratio-intensity maps showed the five significantly different proteins at 1817.7, 2010.7, 2710.2, 2744.8, and 4134 Da, which had a particular trend among the four groups. Green curve, no-appliance group; orange curve, 2-month group; red curve, 7-month group; blue curve, 12-month group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Significant ($p < 0.05$) *m/z* values identified in multiple comparisons.

Group (A *vs.* B)*	Mean *m/z* value	*P*-value	Tendency**
0 *vs.* 2	2744.8	1.95E-04	↑
	2010.7	0.007	↑
	4134	0.016	↓
	2710.2	0.022	↑
	1817.7	0.03	↑
	4811.9	0.038	↑
0 *vs.* 7	2710.2	0.005	↑
	4134	0.015	↓
	3419.3	0.017	↑
	1817.7	0.025	↑
	2010.7	0.028	↑
	2744.8	0.030	↑
2 *vs.* 12	2010.7	0.013	↑
	1817.7	0.027	↑
	1423.3	0.037	↓
	2744.8	0.045	↑
	2710.2	0.045	↑
	4134	0.048	↓
7 *vs.* 12	2710.2	0.025	↑
	1817.7	0.035	↑
	4134	0.037	↓
	1423.3	0.045	↑
	2744.8	0.048	↑
0 *vs.* 12	2481.4	0.002	↑
	2010.7	0.002	↑
	1817.7	0.003	↑
	1306.1	0.009	↑
	4134	0.013	↓
	3524.4	0.026	↓
	2710.2	0.035	↑
	2223	0.045	↑
	2744.8	0.045	↑

* A and B refer to treatment duration (in months) in each group.
** Tendency refers to the trend in the intensity of *m/z* values between the two groups: ↑, higher intensity in group B than in group A; ↓, lower intensity in group B than in group A.

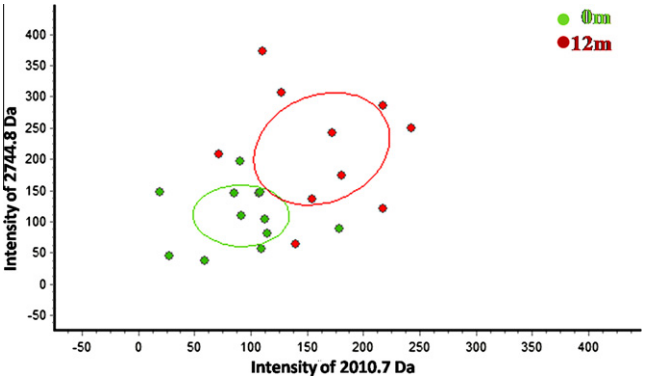

Fig. 4. Scatter plots of the no-appliance and 1-year groups established by combining proteins 2010.7 and 2744.4 Da and showing a well-distinguished fitting shape of the curve.

mass spectra are easy to interpret [23]. MALDI-TOF MS was used in combination with WCX here to initially select peptides in the range of 1000–10,000 Da prior to further identification using other techniques. The effectiveness of this combination of techniques has been confirmed in many serum-based peptide profile identification studies [24,25]. MALDI-TOF MS generated an accurate protein profile in patients with fixed orthodontic appliances at different treatment time points from small saliva samples.

Some studies have indicated that orthodontic procedures contribute to changes in saliva condition, with an emphasis on the physical or chemical behaviors of saliva [26], microbial activities [27], metal ions released by the orthodontic appliances [28], or pain intensity after appliance bonding [29]. However, proteomic/peptidomic studies related to orthodontic treatment duration have not been well performed, and biomarker information remains unclear. Therefore, further investigations of more informative proteins are essential to understand the inner physical or pathological mechanism.

Among the eight significantly different peaks, the five peaks (1817.7, 2010.7, 2710.2, 2744.8 and 4134 Da) that presented steady trends suggested that protein expression changed regularly as orthodontic treatment progressed. Moreover, after the mass for the MS/MS spectrum were acquired for the peptides, their amino acid sequences were determined by matching the MS/MS spectrum to a known in silico-generated database of peptide spectra using search algorithms, such as SEQUEST, which was plotted for no-enzyme (unconstrained) searches of the IPI and/or NR database in an uninterpreted manner [30].

Interestingly, apoE, which was matched to peptide 2010.7 Da, has been established as a novel regulator of bone metabolism in mice. ApoE deficiency in mice leads to a high bone mass phenotype and modifies the effect of pathophysiological conditions, such as hyperlipidemia, obesity and renal insufficiency on bone [31]. The significance of the prediction was that these differential expression patterns may have originated from the distinctly complex conditions of bone metabolism or the inflammation related to alveolar bone remodeling, tooth movement, or root resorption. However, it must be kept in mind that a peptide sequence usually does not exclusively define a single protein. Ultimately, the aim of the investigation was to determine the protein or gene from which a peptide is derived; this is not easy but complex. As the constituents showed distinct levels according to treatment duration, these biomarkers could be useful for determining the precise force and duration that should be applied in patients. This would ultimately lead to an optimal effect with minimal side effects and could accelerate treatment progress.

The present study demonstrated that screening for saliva protein profiles shows differential expression in mass spectra peak intensities among the four groups according to different orthodontic treatment durations. With the same inclusion criteria and the patients receiving similar orthodontic appointment, the different treatment durations could account for the differential peptide expressions. It showed statistical differences when compared 2, 7, 12 months groups, which performed consistent age. We could find statistical difference between the 2 and 12 months groups at all the five peptides, the comparison between 7 and 12 months groups also showed statistical difference at peptides 1817.7, 2710, 2744.8 and 4134 Da. However, the average age in the no-appliance group differed from that in the other three groups because of sample collection difficulties. When the four groups were compared, peak intensity in 0 month group significantly differs from those in the other groups. It seemed to indicate that the significant differences among the four groups did not exclusively originate from the changes of peptidome profile during orthodontic treatment, as age of 0 month group did not consist with the other three groups. It still needs further exploration that whether and how age might in some sense contribute to the differential peptide expression.

In subsequent work, we will apply the potential biomarkers to additional groups to establish a concise monitoring model for orthodontic treatment. The analysis of saliva is inherently challenging because it contains a large number of proteins within a particularly wide concentration range [7] that could be modified post-translationally [32]. However, it is likely that the use of saliva will continue to expand, and that it will provide a new and promising tool to investigate physiological and pathophysiological states.

In conclusion, our study indicated that the peptide profiles changed as the orthodontic treatment proceeded by using

magnetic bead-based MALDI-TOF MS. Thus, this method provides a new tool to analyze the mechanism of orthodontic tooth movement, and would ultimately lead to an optimal effect and might accelerate treatment progress. However, expanding the data set of orthodontic patients and further identification of the biomarkers, will help establish a concise monitoring model for orthodontic treatment.

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